CHARACTERIZATION AND QUALITY IMPROVEMENT OF MNH992 AND VH289 BT COTTON SEED VARIETIES

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ABSTRACT

In this study, we used PCR and ELISA techniques to identify the specific Cry1Ac gene and proteins in MNH992 and VH289 transgenic cotton plants. MNH992 was extracted at a concentration of 21.9ug/g and VH289 at a concentration of 17.37ug/g from seed protein. PCR analysis confirmed that the gene specific CRY1Ac F/R primer produced amplicons with sizes of 550bp for the Bt gene, 600bp for the nptII gene, and 200bp for the 35s promoter gene. The ELISA method was used to validate the developed assay because it required less equipment and took less time.

Keywords: PCR; ELISA; MNH992; VH289; Cry1Ac; Cotton.

INTRODUCTION

The purpose of this study was to develop an immuno-PCR technique for detecting toxin levels in bt cotton seeds. Globally, the area cultivated Bt cottons expressing Cry1Ac gene expanding year after year [1]. The most significant event has been the adoption of Bt transgenic cotton hybrids in India and efficient detection strategies for genetically modified crops must be in compliance with regulatory frameworks [2]. Because of their ease of use and accuracy in detecting GM and non-GM crops, DNA-based techniques such as PCR, RT-PCR, LAMP, RT-LAMP, and Multiplex PCR are currently most commonly used detection methods [3]. RFLP was the first widely reported method of revealing DNA sequence variations in a wide variety of organisms, including varieties, and its utility for variety identification has been confirmed [4]. RAPD and AP-PCR were widely used to fingerprint various plant species, but were failed in reproducibility across laboratories and a lack of polymorphisms in some important crops such as wheat [5]. Bacillus thuringiensis is a naturally
occurring bacterium that produces proteins that are lethal to insect larvae [6]. By transforming the genes that encode these proteins into cotton balls, scientists have created a type of cotton that produces its own pesticides, making it resistant to insects [7]. Bt cotton plant produces the Cry1Ac insect-control protein and the nptII selectable marker protein [8]. The availability of unapproved seeds in the market has been the reason for controversies regarding the acceptance among the farmers made a need for GM detection and testing [9]. Analytic techniques are frequently determined by the quantitative and qualitative characteristics of the target analyte, and conventional PCR is widely used for this purpose due to its specificity, sensitivity, and dependability [10]. The findings of this research support the recommendation to improve the existing quality criteria for the approval and certification of transgenic cotton varieties [11,12].

MATERIALS AND METHODS

Collection of Seed Material

Cotton seed samples of MNH992 and VH289 were obtained from a retailer shop in Guntur, Andhra Pradesh, for the purpose of characterization and identification of the Cry1AC gene [13].

Preparation of Seed Smoothie

Cotton seeds were cleaned thoroughly with distilled water to remove contaminants and dust and were made into a smoothie with the help of a mortar and pestle and collected into a sterile test tube [14]. These tubes were centrifuged at 10,000 rpm for 3-5 minutes. The supernatant was separated and stored for analysis against blood samples.

DNA Extraction

Genomic DNA was extracted from cotton seeds incubated with liquid nitrogen by CTAB method [15]. Extracts were placed in water bath (with gentle shaking) for 60 minutes at 65°C with periodical shaking at an interval of five minutes. 10 ml of Chloroform: Isoamyl alcohol mixture (24:1) was added to extract and the contents were mixed by shaking then the tubes were centrifuged for 10 minutes at 10,000 rpm at room temperature. Equal quantity of chilled isoproponal was added to each tube and mixed by inverting and incubated at -20°C for overnight. The content was centrifuged for ten minutes at 10,000 rpm at 4°C. The supernatant was discarded. The DNA pellet obtained was washed with 70 percent ethanol and the tubes were inverted on blotter paper to dry the pellet. The DNA was dissolved in 100μl TE buffer and stored at -20°C for further study. The quality and quantity of DNA were estimated using a spectrophotometer based on the 260/280-nm and 260/230-nm UV absorption ratios and analyzed by 0.8% agarose gel electrophoresis.

PCR Analysis

PCR analysis of transgenic cotton was carried out by using the primer Cry1Ac F/R [16]. The primer sequence and their amplicon size are shown in Table 1 synthesized from Bioserve Biotechnologies, Hyderabad. PCR amplification was carried out by using 25μl reaction mixtures containing Taq DNA polymerase (Genei, Bangalore) 25μM of each forward and reverse primers, 200 mM of each dNTP and 3μl DNA. The master mix of 25μl was added to PCR tubes and was given a short spin to mix the contents. The tubes were placed in the thermal cycler for amplification with cycling conditions: Denaturation: 94°C for 30 sec, Annealing: 50°C for 50 sec, Primer extension :72°C for 30 sec for 35 cycles. PCR product was subjected to gel electrophoresis containing 2% agarose gel and bands were captured under UV light (254-366 nm) by UV- Gel Documentation System (UVI-Tech, Germany).

Analysis of Cry1AC protein by ELISA

A 96-well microtiter plate precoated with anti-Cry1Ac antibodies from Thermo was used. On one side, 50μl of positive control Cry1Ac (Thermo) was added to two wells on opposite corners, while on the other side, 50μl of negative control Cry1Ac (Thermo) was added to two wells on opposite corners. The contents were thoroughly mixed by gentle shaking and incubated at room temperature for 30 minutes. Following washing, 100μl of substrate solution (TMB) was added to each well and incubated for another 20 minutes at room temperature. After, 100μl of stop solution
Table 1. Primer used for detection of Cry1AC gene in cotton seed samples

<table>
<thead>
<tr>
<th></th>
<th>Bt</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>NptII</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>35S promoter</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TACTTGTTGGAGAACGCATTGAA</td>
<td>GAGGTCGACTAGTCCGACACGGA</td>
<td></td>
<td>CGCTATGTCCGTAGACGGGTCC</td>
<td></td>
<td>GATAACAGAAATCGGTGCTC</td>
<td></td>
<td>GCTCCTACAAATGCCATCA</td>
<td></td>
</tr>
</tbody>
</table>

(Sulphuric acid) was added to each well immediately. Afterword the absorbance of each well’s content was measured at 450nm by using an ELISA reader (Thermo Multiskan) along with the positive and negative control wells [17].

RESULTS AND DISCUSSION

DNA Extraction

The DNA yield ranged from 26 to 50 mg per 100 mg of fresh tissue. The A260/A280 ratio was found to be 1.89-2.3, indicating that the isolated DNA was protein-free. Fig. 1 depicts a comparison of relative DNA yields obtained from MNH992 and VH289 extractions, as well as a DNA marker (Genei, Bangalore).

PCR Analysis

We used a PCR-based approach to detect, identify, and confirm the gene stability of the cry1Ac transgene construct in Bt cotton. For the BT gene, a 550 bp amplicon was obtained as a PCR product by using Cry1AC primers for MNH992 as shown in figures 2 and 3 and nptII primers for VH289 as shown in Figs. 4 and 5.

Analysis of Cry1AC protein by ELISA

The interpretation of results was done based on the mean absorbance data of the individual samples. The cutoff value for Cry1Ac protein presence or absence in the sample was 1.0. Samples with absorbance less than 1.0 were considered negative, while samples with absorbance greater than 1.0 were considered positive for the presence of Cry1Ac protein. Based on the research so far, it can be assumed that the ELISA technique shown in figure 6 for identifying Cry1Ac protein in the sample is quite useful and simple to implement.

Fig. 1. Agarose gel electrophoresis of genomic DNA extracted from MNH992 and VH289 variants of cotton seeds, M-DNA marker, Lanes 1 & 2 DNA from MNH992 and Lane 3 DNA from VH289
Fig. 2. PCR amplicon patterns for MNH992 generated by Cry1AC primers. M-100 base pair molecular weight marker, Lanes 1 to 65 are BT cotton samples of MNH992. Lanes 6, 13, 26, 29, 33 were found to be negative. (An amplicon of 550 bp was not generated in these lanes)

Fig. 3. PCR amplicon patterns for VH289 generated by Cry1AC primers. M-100 base pair molecular weight marker, Lanes 1 to 65 are BT cotton samples of VH289. Lane 52 was found to be negative. (An amplicon of 550 bp was not generated in these lanes)

Fig. 4. PCR amplicon patterns for MNH992 and VH289 generated by nptII primers. M-100 base pair molecular weight marker, Lanes 1 to 17 are BT cotton samples of MNH992 and RCH 2 respectively. By using primers for nptII promoter approximately a 600 bp amplicon was obtained as PCR product
CONCLUSION

The genetic resistance is the economical and the most efficient method of protecting crop plants from pests. Transgenic cottons with Bt endotoxin protein do reduce expenditure on insecticides and create eco-friendly environment without reduction in yield. Both PCR-based and ELISA-based screening for the purity of commercial Bt Cotton hybrids were successful. The results show that commercial Bt Cotton seeds on the market are not 100 percent pure. Based on the above findings, it is possible to conclude that VH289 samples are purer than MNH992. It can also be concluded that the technology used in the current study is ideal for regular screening of Bt Cotton hybrid seed and benefit the use of seeds by farmers for high yield.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

Fig. 5. PCR amplicon patterns for MNH992 and VH289 generated by 35s promoter primers. M-100 base pair molecular weight marker, Lanes 1to 17 are BT cotton samples of MNH992 and RCH 2 respectively. By using primers for 35S promoter approximately a 200 bp amplicon was obtained as PCR product. Lane 14 of RCH 2 was found to be negative & remaining are positive

Fig. 6. In the seed samples of VH289 the amount of delta endotoxin was found to be 12.87 – 17.347 μg per gram of tissue. In the seed samples of MNH992 the amount of delta endotoxin was found to be 19.15 – 21.19 μg per gram of tissue
COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES